DIGESTIVE ENZYMES OF THE CRYSTALLINE STYLE OF STROMBUS GIGAS LINNE.[1] I. CELLULASE AND SOME OTHER CARBOHYDRASES

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The crystalline style is a flexible hyaline rod composed of mucoprotein gel. Among marine molluscs it occurs in most lamellibranchs and in a few gastropods. The digestive enzymes of the crystalline style are set free into the gut lumen by dissolution of the style as it is rotated against a cuticular gastric shield by the cilia of the style sac epithelium. Yonge (1932) pointed out that *Strombus gigas,* feeding on fine filamentous algae, was the largest of herbivorous gastropods. Yonge's observations were later confirmed by Robertson (1961) and by Randall (1964), who showed that this conch feeds unselectively on delicate macroscopic algae, on unicellular algae, and on algal detritus. Microscopic examination of the stomach contents of *Strombus gigas* Linné from the Miami area has confirmed the ability of this animal to digest cell walls of filamentous green algae and to dissolve algal cytoplasm. These changes were particularly marked in green algae adherent to the surface of the head of the crystalline style.

A cellulase enzyme system would facilitate use of algal cellulose by *S. gigas.* Cellulolytic activity has been demonstrated in the crystalline style of the clams, *Mya* and *Mactra* (Lavine, 1946), an oyster, *Ostrea* and a mussel, *Mytilus* (Newell, 1953), an African bivalve, *Caelatura* and a marine snail, *Melanoides* (Fish, 1955), the wood-boring pelecypods, *Bankia* (Nair, 1955, 1957) and *Teredo* (Greenfield and Lane, 1953), and the lamellibranchs, *Cardium* and *Scorbicularia* (Stone and Morton, 1958). During feeding experiments Dean (1958) observed that algal cells of *Cryptomonas* were destroyed while this alga was swimming near the style of an oyster, *Crassostrea.* In this paper the cellulase activity of the crystalline style of *S. gigas* will be described. Subsequent papers in this series will examine other enzymes of the crystalline style.

Materials and Methods

Specimens of the queen conch, *S. gigas,* which is native to Southeast Florida and the West Indies, were collected from shallow water adjacent to Virginia Key, Miami, Florida. They were maintained in sea water pens on the laboratory grounds until they were used. The style of *S. gigas* is large; one of the largest in the present series of samples measured 18.5 cm. long, 0.55 cm. wide, and weighed

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2.100 gm. The average moisture content was 82.4% and N averaged 8.19% by a micro-Kjeldahl method. Broken algal fragments and other detritus are often embedded in the substance of the portion of the style (*ca.* 0.5 cm. in length) protruding from the style sac into the lumen of the gut.

The animals were separated from the shell and the style removed quickly. The exposed head of the style was cut off and discarded. The surface of the remainder of the style was scraped with a sharp knife to remove adherent debris. It was then washed by shaking several times in sterile sea water. The styles were then homogenized in a sterile Waring Blendor with sterile 0.66 *M* phosphate buffer (pH 6.55) made isotonic with sea water by the addition of NaCl. This medium was 0.620 *M* NaCl, the same as that used earlier for the extraction of cellulase in *Teredo* (Greenfield and Lane, 1953). The viscous homogenate was stored at 4° C. for 12 hours, then centrifuged at 20,200 *g* at 4° C. for 30 minutes. The small residue was discarded. The supernatant solution was lyophilized, and the resulting powder was stored at − 10° C. There was no significant loss of cellulolytic activity during three months of storage. The powder was dissolved in citric acid-sodium phosphate buffer at room temperature and centrifuged briefly at low speed. The supernatant solution was filtered through a Millipore membrane. The sterility of the resulting enzyme solution was established by broth culture with Difco Tryptic Soy Broth. Cultures were negative for growth at both 24 and 48 hours at 30° C. Control experiments employed the sterile enzyme solution that had been boiled for 10 minutes.

Cellulolytic activity was estimated both by the formation of reducing sugar and viscosimetrically. Reducing sugar was estimated by the methods of Somogyi (1928, 1952) as modified by Nelson (1944). Optical density was measured at 500 mμ with either the Beckman Model DU spectrophotometer or Coleman Model 6A spectrophotometer. Samples of sodium carboxymethyl cellulose (CMC) from Hercules Powder Company, of different degrees of polymerization (D.P.) were used as substrates in the cellulase assay. Purified sodium alginate (Fisher Scientific Company), and carrageenan (Marine Colloids, Inc.) were also employed. In testing for cellobiase, glucose was estimated by the Glucostat Special reagent (Worthington Biochemical Corp.). The pH of the reaction mixture was determined with a Beckman glass electrode before and after incubation. Digestive activity was expressed as μg of reducing sugar liberated per milligram of dry style extract per hour.

Changes in substrate viscosity were measured in Ostwald viscosimeters. Three ml. of substrate solution (CMC to final concentration 0.8%) were mixed in the viscosimeter with 3.0 ml. of McIlvaine's citric acid-sodium phosphate buffer (pH 6.75). After thermal equilibrium in the 35° C. water bath had been achieved, the time required to empty the capillary was noted. One-tenth ml. of enzyme solution was added and mixed in a stream of air bubbles. The time required to empty the capillary was measured at intervals from the time of mixing.

Results

Filtration through Millipore membranes does not affect the activity of style enzyme solutions as judged by two different criteria (Tables I and II). The yields of reducing sugar from CMC 70 of three different degrees of polymerization in-

TABLE I

*Hydrolytic activity of crystalline style extract before and after bacterial filtration**

Substrate	Unfiltered enzyme solution	Millipore-filtered enzyme solution	Boiled enzyme solution
CMC 70 M	90.5 ± 9.0	89.7 ± 10.3	11.7 ± 1.7
Phosphoric acid-swollen cellulose	10.3 ± 0.7	10.9 ± 1.1	3.8 ± 0.4
Cellulose powder	2.1 ± 0.3	1.9 ± 0.3	0.7 ± 0.2
Sodium alginate	5.7 ± 0.3	6.1 ± 0.5	0.6 ± 0.3
Carrageenan	2.4 ± 0.7	2.7 ± 0.5	0.7 ± 0.2
Cellobiose	8.9 ± 0.5	8.7 ± 0.5	3.7 ± 0.4

* Activity expressed as μg. reducing sugar/mg. crystalline style powder/hour.
Each value is the mean and standard deviation of 10–17 determinations.
Reactants incubated at 35–37° C. at pH 6.75.

cubated with crystalline style enzyme solutions are shown in Table III. This table shows that different degrees of substitution in the CMC substrate had only a slight effect on the activity of the enzyme preparation. These results differ somewhat from those reported for *Limnoria* by Ray (1959). In this form maximum cellulase activity was found when CMC 70 High was the substrate. When the cellulase activity of *S. gigas* style extract was estimated viscosimetrically, however, the results, shown in Figure 1, more nearly resemble those reported by Ray. Whatman No. 1 filter paper, swollen in 85% phosphoric acid and suspended at 0.5% concentration in phosphate buffer and the same concentration of Whatman cellulose powder, was also used as a substrate. These results also appear in Table I. The style enzyme solution is less effective on these substrates than on the CMC samples used. Figure 2 shows the linear relationship between cellulase activity and enzyme concentration measured by protein nitrogen. The relationship between pH and cellulolytic activity is shown in Figure 3. There is a single peak of activity between pH 6.8 and pH 7.2. When determined viscosimetrically after 60 minutes incubation the pH optimum was 6.75. The pH of gut contents of *S. gigas* ranged from 6.25 to 6.65.

TABLE II

Viscosity of enzyme-substrate before and after bacterial filtration*

Substrate		Incubation time				
		15 min.	30 min.	60 min.	90 min.	120 min.
CMC 70 M	I	67.8 ± 1.4	54.7 ± 1.2	44.9 ± 2.0	39.6 ± 2.2	36.5 ± 2.4
	II	74.2 ± 3.1	62.9 ± 3.2	52.1 ± 3.3	47.4 ± 2.7	44.5 ± 1.8
Sodium alginate	I	89.0 ± 3.2	80.9 ± 2.8	67.7 ± 2.1	58.2 ± 2.9	52.3 ± 2.8
	II	88.1 ± 2.2	82.2 ± 3.8	72.0 ± 3.2	63.9 ± 3.2	56.7 ± 2.7
Carrageenan	I	90.9 ± 1.9	83.3 ± 2.5	74.7 ± 2.2	68.4 ± 1.8	64.3 ± 2.2
	II	90.7 ± 5.8	86.9 ± 0.1	79.8 ± 0.6	74.2 ± 0.4	70.7 ± 0.1

* Viscosity is expressed as time in seconds for capillary emptying.
I = Unfiltered enzyme solution.
II = Millipore-filtered enzyme solution.

TABLE III

Cellulase activity of the crystalline style of Strombus gigas with various substrates

Substrate	D.S.	Viscosity	μg. glucose/mg. of enzyme/hr.
CMC 70 low	0.82	30 cps.	31.4
CMC 70 med.	0.77	470 cps.	41.1
CMC 70 high	0.72	2100 cps.	15.0
CMC 90 high	0.94	280 cps.	17.8
CMC 120 high	1.25	135 cps.	7.8
Swollen filter paper			3.2
Cellulose powder			0.7

Incubation at 30° and pH 7.4 for CMC, pH 7.3 for other substrates.

The optimum temperature and the temperature of inactivation of the enzyme solution were determined. The relationship between the incubation temperature and cellulase activity showing an optimum about 40° C. is presented in Figure 4. Thermal stability of the enzyme solution was determined by assaying residual activity after heating the enzyme solution to various temperatures for fifteen minutes

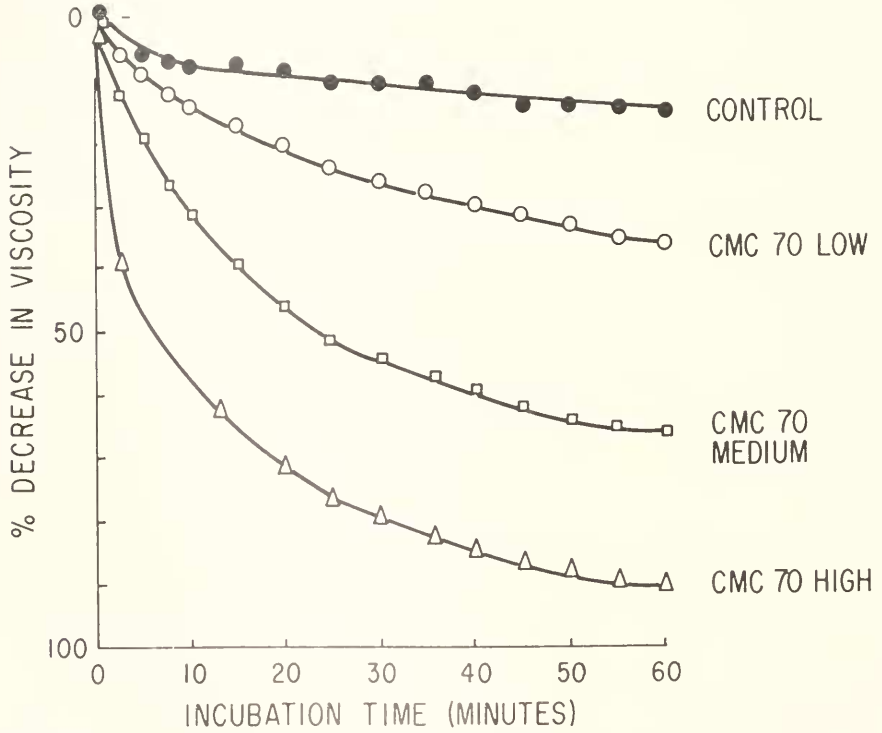

FIGURE 1. Effect of crystalline style enzyme on CMC 70 of different viscosity. Final concentration of CMC 70 was 0.8%; pH was 6.75 and temperature 35° C.

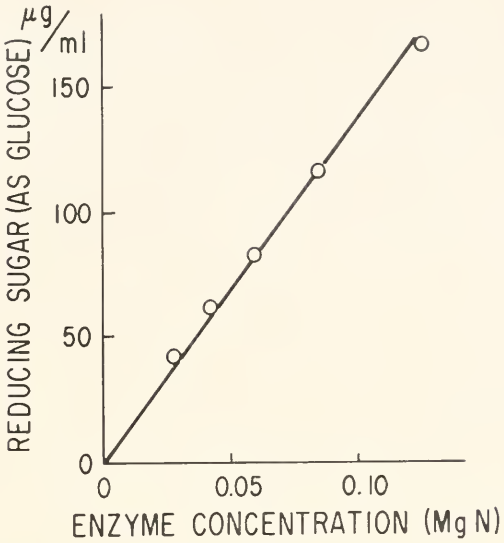

FIGURE 2. Cellulase activity of crystalline style extract at various enzyme concentrations. Reaction mixtures containing 12 mg. CMC 70 of medium viscosity were incubated at 30° C., pH 6.8 for two hours.

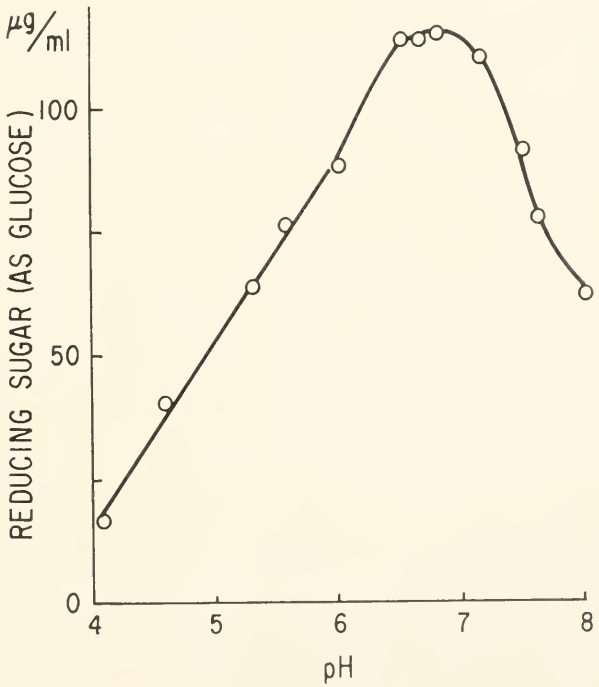

FIGURE 3. Cellulase activity of the crystalline style at various pH levels. Substrate was CMC 70 of medium viscosity; temperature was 30° C.; time was two hours.

(Fig. 5). Activity was undiminished up to 45° C. Between 45° C. and 50° C., cellulolytic activity was markedly reduced and was 90% destroyed at 70° C.

Cellobiase activity (Table IV) was estimated by incubating cellobiose with crystalline style enzyme solution. Glucose produced was determined by the specific glucose oxidase method (Worthington Biochemical Corp.). As compared with the cellulase activity, the cellobiase activity is slight. Since the crystalline style of *S. gigas* appears to be deficient in cellobiase, it appears that digestion of cellulose by this animal does not necessarily include cellobiose as an intermediate (Levinson and Reese, 1950).

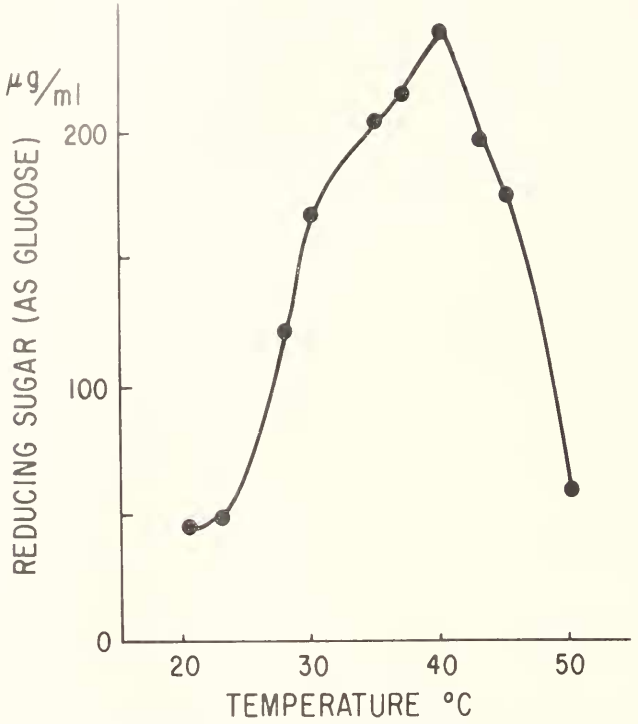

FIGURE 4. Effect of temperature on cellulolytic activity of the crystalline style extract. Substrate was CMC 70 medium; pH 6.75; incubation time was two hours.

The relationship between decreasing viscosity and increasing concentrations of reducing sugar during cellulolysis is presented in Figure 6.

DISCUSSION

There is general reluctance to attribute cellulase enzyme activity to higher Metazoa because symbiotic microorganisms are involved in cellulose breakdown in most higher animals. Indeed, Morton (1952, 1960), Newell (1953) and Barrington (1952) have emphasized the occurrence of spirochaetes in the crystalline style of many bivalves. Some, at least, of the enzymatic capability of the style is attributed to these symbionts.

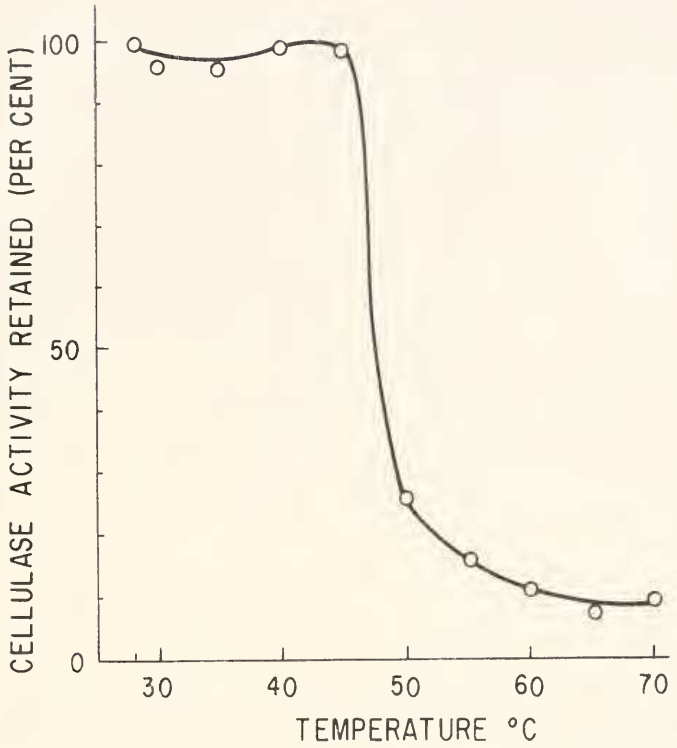

FIGURE 5. Temperature stability of cellulase in the crystalline style. Activity of the heated enzyme, assayed after two hours in a reaction mixture containing CMC 70 medium, at 35° C., pH 6.8.

Levinson and Reese (1950) suggested that at least two kinds of enzymes were involved in the complete degradation of native cellulose. First, a C_1 enzyme causes a rapid decrease in viscosity by converting native cellulose to linear anhydroglucose chains. These are then hydrolyzed to the soluble sugars glucose and cellobiose by C_x enzymes (Gascoigne and Gascoigne, 1960; Levinson and Reese, 1950). Our results strongly suggest that the cellulolytic capability of the crystalline style of *S. gigas,* together with certain other amylolytic activities, are of molluscan rather than bacterial origin. If it be assumed that CMC in solution is a straight-chain molecule made up of 1,4-β-glucose linkages (Levinson and Reese, 1950), then all the

TABLE IV

Cellobiase activity of the crystalline style of Strombus gigas Linné

pH	5.7	6.6	7.6
Glucose (μg.)	16.3	16.2	10.3

0.5% cellobiose in McIlvaine buffer was incubated at 35° C. for three hours. Reaction mixtures contained 1.8 mg. of lyophilized style.

soluble derivatives of cellulose used in this study were hydrolyzed by a C_x enzyme of the crystalline style. Digestion of algal cellulose by *S. gigas* probably includes some preliminary microbiological degradation followed by extracellular digestion by style enzymes in the stomach (Evans and Jones, 1962). Digestion of lower molecular weight sugars and other partially digested foods is probably completed intracellularly in phagocytic cells of the digestive diverticula.

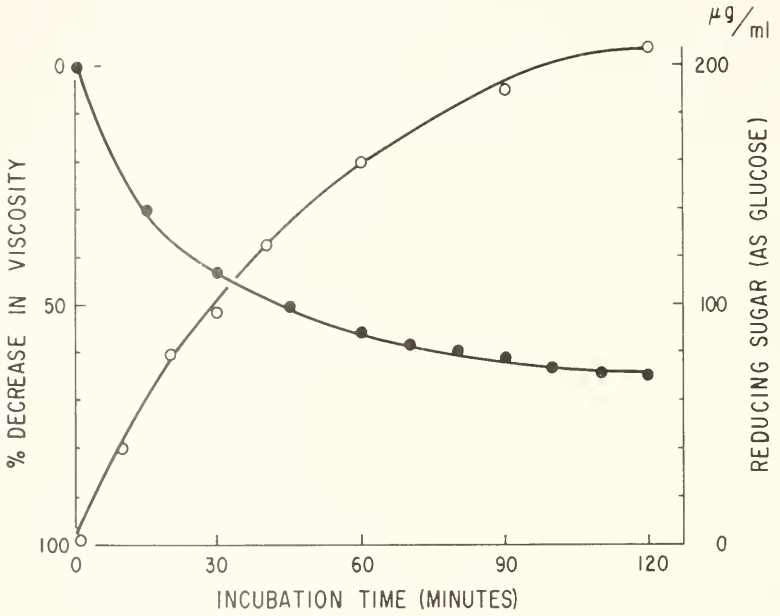

FIGURE 6. The relationship between decrease in viscosity and formation of reducing sugar during cellulolysis by the crystalline style. Reaction mixture contained 0.6% CMC 70 medium viscosity; pH 6.75 at 35° C.

SUMMARY

The crystalline style was extracted in buffered saline and the extract subsequently lyophilized. The activity of the resulting enzyme powder was determined by measuring the amount of reducing sugar it liberated from various substrates under different conditions, and by measuring the decrease in viscosity of these substrates. Cellulase activity was proportional to enzyme concentration. The pH optimum was between pH 6.8 and pH 7.2. Optimum temperature for enzyme activity was 40° C. Between 45° and 50° C., cellulolytic activity was markedly reduced. Cellobiase activity of the style extract was slight. Bacteria-free extracts were as active as unsterile preparations. Some implications of these observations are discussed.

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